Biochemistry

 Volume 25, Number 8 April 22, 1986

Accelerated Publications

Correlation between Sites of Limited Proteolysis and Segmental Mobility in Thermolysin†

Angelo Fontana,* Giorgio Fassina, Claudio Vita, Daniele Dalzoppo, Moreno Zamai, and Marcello Zambonin

Department of Organic Chemistry, Biopolymer Research Centre of CNR, University of Padua, Padua, Italy

Received January 6, 1986; Revised Manuscript Received February 6, 1986

ABSTRACT: Limited proteolysis or autolysis of thermolysin under different experimental conditions leads to fission of a small number of peptide bonds located in exposed surface segments of the polypeptide chain characterized by highest mobility, as given by the temperature factors (*B* values) determined crystallographically [Holmes, M. A., & Matthews, B. W. (1982) *J. Mol. Biol. 160*, 623–639]. Considering also similar findings observed previously with other protein systems, it is proposed that this correlation between segmental mobility and sites of limited proteolysis in globular proteins is quite general. Thus, flexibility of the polypeptide chain of a globular protein at the site of proteolytic attack promotes optimal binding and proper interaction with the active site of the protease. These findings emphasize that apparent thermal motion seen in protein crystals is relevant to motion in solution and appear to be of general significance in protein–protein recognition processes.

A native globular protein is not a static entity but rather one that undergoes conformational fluctuations about its most stable conformation, as clearly emphasized in recent years by numerous studies using a number of varied physical methods (Gurd & Rothgeb, 1979; McCammon & Karplus, 1983). Crystallographic results have provided direct evidence of intramolecular motions in proteins, as indicated by the fact that certain parts of the protein molecule are systematically poorly resolved, e.g., the terminal atoms of long, flexible, external side chains such as that of lysine (Takano, 1977), the ends of polypeptide chains (Thornton & Sibanda, 1983), or some segments within the chains such as segment 18–23 in ribonuclease S (Richards & Wyckoff, 1971, 1973). More clear-cut evidence for motion in proteins comes directly from highly refined analysis of the diffraction data giving information about mean atom displacements, as usually expressed by the crystallographic temperature factors (*B* values). The *B* value represents the mean-square displacement of each atom and, when plotted against residue number, provides a graphic image of the degree of mobility existing along the polypeptide chain. In particular, it has been found that *B* values are, in general, low in helical regions and high at bends and chain termini (Frauenfelder et al., 1979; Artymiuk et al., 1979; Sternberg et al., 1979).

We will show in this paper that the sites of the polypeptide chain characterized by highest mobility are the more susceptible ones to proteolytic attack. This study was carried out with thermolysin, the highly thermostable metalloendoprotease from *Bacillus thermoproteolyticus* (Endo, 1962), for which the amino acid sequence of its chain of 316 amino acid residues (Titani et al., 1972) and three-dimensional structure (Matthews et al., 1972, 1974; Colman et al., 1972) are known. More recently, a highly refined structure of thermolysin at 1.6-Å resolution has been reported, together with the average thermal motion (segmental mobility) of its polypeptide chain (Holmes & Matthews, 1982). To perform limited proteolysis of thermolysin, subtilisin was chosen as the attacking protease, or autolytic degradation was used. Because of the broad specificity of both subtilisin (Harris & Roos, 1959) and thermolysin (Morihara & Tzusuki, 1970; Keil, 1982), it was anticipated that peptide bond fission would occur at sites dictated by the stereochemistry and flexibility of the polypeptide substrate, and not by the specificity of the protease.

Materials and Methods

Thermolysin from *Bacillus thermoproteolyticus* (Rokko) was obtained from Sigma Chemical Co. (St. Louis, MO) as

† This study was supported by the Consiglio Nazionale delle Ricerche, special program on Biotechnology.

0006-2960/86/0425-1847$01.50/0

a lyophilized product containing 30% calcium and sodium acetate. The enzyme was purified by affinity chromatography using Sepharose–Gly-D-Phe (Pangburn et al., 1979). The materials used for SDS–PAGE[1] were obtained from Bio-Rad (Richmond, CA). Ultrapure Gdn·HCl and trifluoroacetic acid (Sequanal grade) were purchased from Pierce Chemical Co. (Rockford, IL). Ultrogel AcA-54 was obtained from LKB (Bromma, Sweden) and Sephacryl S-200 from Pharmacia (Uppsala, Sweden).

SDS–PAGE was carried out with a vertical slab gel apparatus (Laemmli, 1970). An exponential gradient from 15 to 24% along the direction of migration was used. The gels (1-mm thickness) were stained with Coomassie Brilliant Blue R-250. The experimental conditions used for amino acid analyses, Edman degradations, and identification of phenylthiohydantoin derivatives of amino acids by HPLC have been described (Vita et al., 1985; Dalzoppo et al., 1985). To determine the carboxyl-terminal sequence, some protein fragments were incubated at 37 °C with carboxypeptidase Y (Sigma) in 0.1 M Tris-HCl buffer, pH 7.2, and the time–course release of amino acids was determined by the use of the amino acid analyzer.

The experimental details of the preparation of thermolysin S, the nicked partially active derivative of thermolysin obtained by limited proteolysis by subtilisin Carlsberg, and the isolation and characterization of the two constituting protein fragments [5–224(225)] and [225(226)–316] have been described (Vita et al., 1985). The experimental conditions of the autolysis of thermolysin by heat or EDTA treatment are indicated in the legend to Figure 1. In each case, the proteolysis/autolysis mixtures (10–15 mg of protein) were poured on an Ultrogel AcA-54 column (3.7 × 80 cm) equilibrated with 20 mM Tris-HCl buffer, pH 9.0, containing 10 mM $CaCl_2$ (subtilisin digestion and thermal autolysis), or with 10 mM Tris-HCl buffer, pH 7.2, containing 1 mM EDTA (EDTA-mediated autolysis).

In order to isolate protein fragments, solution samples of nicked thermolysin (as obtained after gel filtration) were poured dropwise with stirring into 10% aqueous formic acid solution. The mixture was lyophilized, and the residue was dissolved in 2 mL of 5 M Gdn·HCl and 50 mM sodium acetate, pH 5.0, and applied to a Sephacryl S-200 column (1.8 × 80 cm) equilibrated and eluted with this Gdn·HCl solution. The effluent was monitored at 280 nm for protein content. Fractions containing peptide material were combined, dialyzed against 2% aqueous formic acid (Spectrapor membrane, molecular weight cutoff of 3500), and then lyophilized. Alternatively, thermolysin fragments contained in solutions of nicked thermolysin obtained after gel filtration were purified to homogeneity by using an HPLC system obtained from LKB consisting of two Model 2150 pumps, a Model 2152 controller, an Uvicord SD Model 2158 ultraviolet detector, and a Model 2210 two-pen recorder. A reverse-phase μBondapak C_{18} column (3.9 × 300 mm) (Waters Associates, Milford, MA) was used with a gradient of water–acetonitrile containing 0.1% trifluoroacetic acid. Protein fragments were monitored in the effluent at 280 and 226 nm and collected manually. For additional experimental details on the methods employed for protein fragment isolation and characterization, refer to previous publications from this laboratory (Vita et al., 1985; Dalzoppo et al., 1985).

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

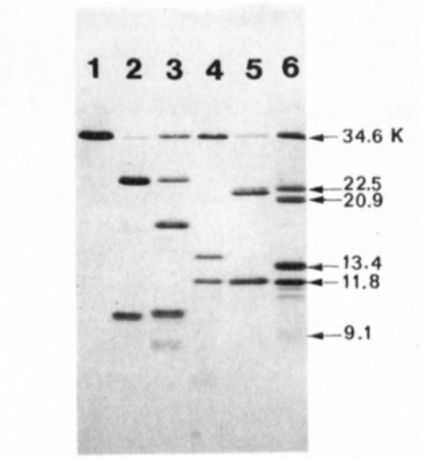

FIGURE 1: Limited proteolysis or autolysis of thermolysin monitored by SDS–PAGE of digestion products. Samples (10 μL containing ~5 μg of protein) were taken from the reaction mixtures, mixed with *o*-phenanthroline (5 μL of a 0.1 M solution in water) to inhibit thermolytic activity (Latt et al., 1969), and then mixed with the sample buffer of the electrophoresis system. Lane 1: Thermolysin dissolved (1 mg/mL) in 20 mM Tris-HCl buffer, pH 7.2, containing 10 mM $CaCl_2$, and incubated for 24 h at room temperature. Lane 2: Limited proteolysis (24 h, 37 °C) of thermolysin dissolved (0.8 mg/mL) in 50 mM Tris-HCl buffer, pH 9.0, containing 10 mM $CaCl_2$ and 5 mM *o*-phenanthroline, by subtilisin (2:100 w/w, molar ratio). Lane 3: Thermal autolysis of thermolysin (60 h, 55 °C) dissolved (0.65 mg/mL) in 50 mM Tris-HCl buffer, pH 9.0, containing 10 mM $CaCl_2$. Lane 4: Autolysis of thermolysin (2 days at room temperature) dissolved (0.65 mg/mL) in 10 mM Tris-HCl buffer, pH 7.2, containing 0.1 M NaCl, 1.5 mM $CaCl_2$, and 1 mM EDTA. Lane 5: Autolysis of thermolysin (20 h at room temperature) dissolved (0.7 mg/mL) in 10 mM Tris-HCl buffer, pH 7.2, containing 0.1 M NaCl, 1.5 mM $CaCl_2$, and 10 mM EDTA. Lane 6: Partial chemical cleavage of thermolysin with cyanogen bromide in 70% aqueous formic acid (100 equiv of reagent/mol of protein, 50 h at room temperature) at the level of the two methionine residues in positions 120 and 205 of the polypeptide chain of 316 amino acid residues (Titani et al., 1972; Vita et al., 1979, 1984). In the right part of the figure are indicated molecular weights of the cyanogen bromide fragments of thermolysin (34.6K), in the order 1–205, 121–316, 1–120, 206–316, and 121–205.

Results and Discussion

Thermolysin was subjected to limited proteolysis (Linderstrøm-Lang, 1949) by subtilisin at 37 °C or to autolysis at 55 °C (60 h) or in the presence of EDTA under the different experimental conditions outlined in the legend to Figure 1. Apparently, under these conditions of mild heating or partial depletion of protein-bound ions by EDTA (Roche & Voordouw, 1978), the structure of thermolysin is slightly perturbed and/or more relaxed, becoming a more suitable substrate for both subtilisin and native, intact thermolysin. The results of the SDS–PAGE analysis (Laemmli, 1970), shown in Figure 1, indicate that under these specified conditions thermolysin is cleaved very selectively at only a few peptide bonds, giving rise to a few fragments of relatively high molecular weight. A cyanogen bromide digest of thermolysin at the two methionine residues at positions 120 and 205 of the polypeptide chain (Titani et al., 1972) served as a marker of the size of the fragments thus obtained (Figure 1, lane 6). From the proteolytic mixtures, stable two- (subtilisin and heat cleavage) as well as three- (heat and EDTA cleavage) fragment complexes were isolated by gel filtration on an Ultrogel AcA-54 column. The complexes ("nicked" thermolysin species) eluted from the column at the same position as intact thermolysin. In the case of thermal autolysis, two- as well as three-fragment complexes were formed; the last eluted from the gel filtration column before intact thermolysin, indicating the presence of an aggregate of higher molecular weight, likely a dimeric

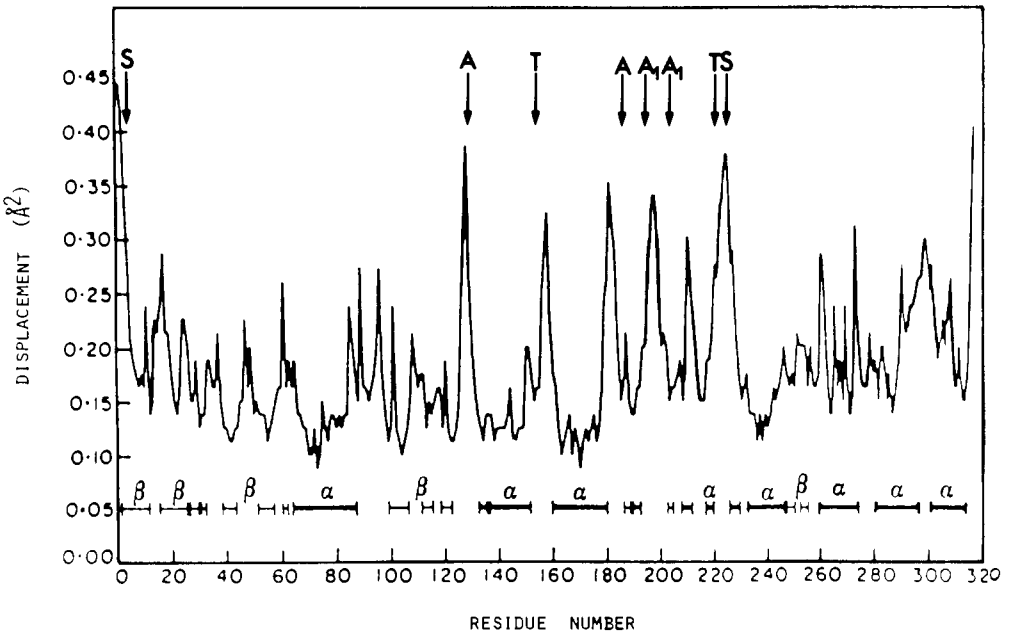

FIGURE 2: Plot of the average main-chain temperature factors (solid line) along the polypeptide chain of thermolysin [adapted from Holmes and Matthews (1982)]. Bars at the bottom of the figure indicate segments of secondary structure (helices and strands). Arrows indicate sites of limited proteolysis or autolysis of thermolysin observed under different experimental conditions, as determined by isolation and characterization of thermolysin fragments. S, site of cleavage of the thermolysin polypeptide chain by subtilisin; A, cleavage by autolysis in the presence of 1.5 mM $CaCl_2$ and 1 mM EDTA; A_1, cleavage by autolysis in the presence of 1.5 mM $CaCl_2$ and 10 mM EDTA; T, cleavage by thermal autolysis.

species. Of interest, the nicked thermolysin species were obtained in high yield (70–80%) after the gel filtration step, indicating that limited proteolysis occurred very selectively at a few specific peptide bonds. The SDS–PAGE analysis of the thermolysin complexes isolated after gel filtration gave a pattern essentially identical with that observed before column chromatography, clearly establishing that fragments remained associated in stable complexes.

The nicked thermolysin species were folded into stable three-dimensional structures very similar to that of intact thermolysin, as indicated by far-ultraviolet circular dichroism measurements and by the fact that all species recognize and precipitate rabbit anti-thermolysin antibodies [unpublished results; see also Vita et al. (1985)]. Apparently, all the nicked thermolysin species described above were inactive when N^{α}-(furylacryloyl)glycyl-L-leucine amide was used as substrate (Feder, 1968), with the exception of the two-fragment complex [fragments 5–224(225) and 225(226)–316] (see below) obtained by limited proteolysis with subtilisin; this species (thermolysin S) maintains ~3% of the catalytic activity of intact thermolysin (Vita et al., 1985).

In order to locate the sites of proteolytic cleavage, the nicked species were first denatured by dissolving them in solutions of 10% aqueous formic acid and then poured on a Sephacryl S-200 column equilibrated and eluted with a 5 M Gdn·HCl solution. Fragments were eluted from this column according to their size and recovered after dialysis and lyophilization. Alternatively, mixtures of proteolytic fragments were isolated by reverse-phase HPLC using a μBondapak C_{18} column eluted with an aqueous acetonitrile gradient containing 0.1% trifluoroacetic acid. A number of peptide fragments were thus purified to homogeneity. The identity of the isolated peptides was established by amino acid analysis after acid hydrolysis, by determination of their NH_2-terminal sequences after three steps of Edman degradation, as well as COOH-terminal sequences, and by determination of the time–course of the release of amino acids during carboxypeptidase Y digestion [for details on methods see Vita et al. (1985) and Dalzoppo et al. (1985)].

Table I: Sites of Limited Proteolysis or Autolysis of Thermolysin[a]

expt no.	experimental conditions[b]	protein fragments isolated	peptide bonds cleaved[c]
1	digestion with subtilisin	5–224(225)	Thr^{4}–Ser^{5}
		225(226)–316	Thr^{224}–Glu^{225}
			Glu^{225}–Asp^{226}
2	thermal autolysis	1–221	Tyr^{221}–Thr^{222}
		1–154(155)	Gly^{154}–Leu^{155}
		155(156)–221	Leu^{155}–Ile^{156}
		224–316	Gly^{223}–Thr^{224}
3	autolysis (1 mM EDTA)	1–129	Thr^{129}–Phe^{130}
		130–187	Glu^{187}–Ile^{188}
		205–316	Ser^{204}–Met^{205}
4	autolysis (10 mM EDTA)	1–196	Gly^{196}–Ile^{197}
		197–204	Ser^{204}–Met^{205}
		205–316	

[a] Thermolysin was subjected to limited proteolysis or autolysis under different experimental conditions, and from the proteolytic mixture stable two- as well as three-fragment complexes were isolated after gel filtration (see text). [b] See legend to Figure 1 for experimental details on the conditions used. [c] The peptide bonds cleaved were established by isolation and characterization of the protein fragments (amino acid analysis after acid hydrolysis, NH_2- and COOH-terminal sequence analysis) and comparison of these data with the amino acid sequence of thermolysin (Titani et al., 1972).

All these data were compared with the known sequence of thermolysin (Titani et al., 1972), and the identities of the peptides originating from the limited proteolysis of thermolysin were unambiguously established. Table I lists the fragments isolated, together with the corresponding peptide bonds being cleaved under the specified conditions of proteolysis.

Figure 2 shows the average mean-square displacement plotted as a function of the thermolysin polypeptide chain (Holmes & Matthews, 1982). In the figure, location of elements of regular secondary structure (α-helix and β-sheet) and of the peptide bonds cleaved by limited proteolysis is indicated. Thermolysin consists of two structural domains of equal size (residues 1–158 and 159–316) (Matthews et al., 1972; Colman et al., 1972; Wetlaufer, 1973), the NH_2-terminal domain

containing mainly β-sheet and being relatively more rigid than the mainly α-helical COOH-terminal domain. The most notable features of the graph of Figure 2 are that highest mobility occurs "in the vicinity of residues 128, 180, and 225, as well as the amino and carboxyl termini" (Holmes & Matthews, 1982), and not in regions of regular secondary structure. The correlation between sites of highest mobility and sites of limited proteolysis appears to be quite striking. For example, thermal autolysis leads to fission between the two structural domains, at peptide bonds 154–155 and 155–156, located between the two helices 137–151 and 160–180, and at the highly flexible peptide loop 220–225. Subtilisin also cleaves at this loop, as well as at the amino terminus. In the presence of EDTA, which removes calcium ions from the metalloprotein, fissions occur at the peptide bond 129–130 and at the loop 180–210, which contains in the native protein the binding sites of three out of four calcium ions (Matthews et al., 1974; Roche & Voordouw, 1978). Cleavage sites are not observed within segments of regular secondary structure (such as helices) but at loops or turns characterized, as expected, by highest flexibility. The majority of cleavage sites occurs within the most extended region of irregular conformation in the thermolysin molecule (region 180–230). An examination of the three-dimensional model of thermolysin indicates that the sites of cleavage (and flexibility) occur at rather exposed loops of the protein molecule (Holmes & Matthews, 1982).

The results of this study put on a firm basis the often made suggestion that limited proteolysis of a globular protein would be expected to occur at surface loops and random segments of polypeptide chains or at flexible hinges between protein domains rather than at internal loci or rigid elements of secondary structure such as helices or pleated sheets [Neurath (1980) and references cited therein]. This suggestion was based on the observed high susceptibility to proteolysis of specific peptide bonds in some globular proteins, such as the hinge peptide of immunoglobulin (Porter, 1959), the 20–22 region of ribonuclease (Richards & Withayathil, 1959), the autolysis loop 142–153 of trypsinogen (Schroeder & Shaw, 1968), and others [see Bennett & Huber (1984) for additional references]. The major result of this study is that it emphasizes that segmental mobility is an essential part of the proteolytic event. One may envision the overall process of proteolysis in which the initial interaction of the globular protein with the protease involves recognition of a specific amino acid sequence of that site, after which some local conformational change takes place in order to make the idealized transition state of the cleavage reaction. Of note, the proteolytic process involves binding, at primary and secondary sites, of peptide segments comprising, on the average, six to eight amino acid residues (Ottesen, 1967; Berger & Schechter, 1970).

Protein–protein interaction is a complex process involving specific characteristics of the interacting molecular surfaces, such as complementarity, hydrophobicity, and electrostatic potential (Blundell, 1981; Katchalski-Katzir, 1983). This study emphasizes the additional role of protein flexibility in protein–protein recognition processes, in general. In this respect, mention should be given to the recently proposed correlation between segmental mobility and the location of continuous antigenic determinants (six to eight residues) in proteins (Westhof et al., 1984; Tainer et al., 1984). In these studies, it has been shown clearly that most continuous antigenic determinants of tobacco mosaic virus, myoglobin, lysozyme, and myohemerythrin are located in surface regions in the protein structure characterized by high segmental mobility.

ACKNOWLEDGMENTS

We thank E. Piaia for expert typing of the manuscript.

Registry No. Protease, 9001-92-7; thermolysin, 9073-78-3.

REFERENCES

Artymiuk, P. J., Blake, C. C. F., Grace, D. E. P., Oatley, S. J., Phillips, D. C., & Sternberg, M. J. E. (1979) *Nature (London) 280*, 563–568.

Bennett, W. S., & Huber, R. (1984) *CRC Crit. Rev. Biochem. 15*, 291–384.

Berger, A., & Schechter, I. (1970) *Philos. Trans. R. Soc. London, B 257*, 249–264.

Blundell, T. L. (1981) in *Structural Aspects of Recognition and Assembly in Biological Macromolecules* (Balaban, M., Sussman, J. L., Traub, W., & Yonath, A., Eds.) pp 281–286, Balaban International Sciences Services, Philadelphia, PA.

Colman, P. M., Jansonius, J. N., & Matthews, B. W. (1972) *J. Mol. Biol. 70*, 701–724.

Dalzoppo. D., Vita, C., & Fontana, A. (1985) *J. Mol. Biol. 182*, 331–340.

Endo, S. (1962) *J. Ferment. Technol. 40*, 346–353.

Feder, J. (1968) *Biochem. Biophys. Res. Commun. 32*, 326–332.

Frauenfelder, H., Petsko, G. A., & Tsernoglou, D. (1979) *Nature (London) 280*, 588–593.

Gurd, F. R. N., & Rothgeb, T. M. (1979) *Adv. Protein Chem. 33*, 73–165.

Harris, I. I., & Roos, P. (1959) *Biochem. J. 71*, 445–450.

Holmes, M. A., & Matthews, B. W. (1982) *J. Mol. Biol. 160*, 623–639.

Katchalski-Katzir, E. (1983) in *Affinity Chromatography and Biological Recognition* (Chaiken, I. M., Wilchek, M., & Parikh, I., Eds.) pp 7–26, Academic, New York.

Keil, B. (1982) *Methods Protein Sequence Anal. [Proc. Int. Conf.], 4th, 1981*, 291–304.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Latt, S. A., Holmquist, B., & Vallee, B. L. (1969) *Biochem. Biophys. Res. Commun. 37*, 333–339.

Linderstrøm-Lang, K. U. (1949) *Cold Spring Harbor Symp. Quant. Biol. 14*, 117–126.

Matthews, B. W., Jansonius, J. N., Colman, P. M., Schoenborn, B. P., & Dupourque, D. (1972) *Nature (London) 238*, 37–41.

Matthews, B. W., Weaver, L. H., & Kester, W. A. (1974) *J. Biol. Chem. 249*, 8030–8044.

McCammon, J. A., & Karplus, M. (1983) *Acc. Chem. Res. 16*, 187–193.

Morihara, K., & Tzusuki, H. (1970) *Eur. J. Biochem. 15*, 374–380.

Neurath, H. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 501–524, Elsevier/North-Holland, Amsterdam and New York.

Ottesen, M. (1967) *Annu. Rev. Biochem. 36*, 55–76.

Pangburn, M. K., Burstein, Y., Morgan, P. M., Walsh, K. A., & Neurath, H. (1979) *Biochem. Biophys. Res. Commun. 54*, 371–379.

Porter, R. R. (1959) *Biochem. J. 73*, 119–127.

Richards, F. M., & Withayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459–1465.

Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes (3rd Ed.) 4*, 647–806.

Richards, F. M., & Wyckoff, H. W. (1973) in *Atlas of Structures for Molecular Biology* (Phillips, D. C., &

Richards, F. M., Eds.) Vol. 1, Oxford University Press, London and New York.
Roche, R. S., & Voordouw, G. (1978) *CRC Crit. Rev. Biochem. 5*, 1–23.
Schroeder, D. D., & Shaw, E. (1968) *J. Biol. Chem. 243*, 2943–2949.
Sternberg, M. J. E., Grace, D. E. P., & Phillips, D. C. (1979) *J. Mol. Biol. 130*, 231–253.
Tainer, J. A., Getzoff, E. D., Alexander, H., Houghten, R. A., Olson, A. J., Lerner, R. A., & Hendrickson, W. A. (1984) *Nature (London) 312*, 127–134.
Takano, T. (1977) *J. Mol. Biol. 110*, 537–568.
Thornton, J. M., & Sibanda, B. L. (1983) *J. Mol. Biol. 167*, 443–460.
Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., & Neurath, H. (1972) *Biochemistry 11*, 2427–2435.
Vita, C., Fontana, A., Seeman, J. R., & Chaiken, I. M. (1979) *Biochemistry 18*, 3023–3031.
Vita, C., Dalzoppo, D., Patti, S., & Fontana, A. (1984) *Int. J. Pept. Protein Res. 24*, 104–111.
Vita, C., Dalzoppo, D., & Fontana, A. (1985) *Biochemistry 24*, 1798–1806.
Westhof, E., Altschuh, D., Moras, D., Bloomer, A. C., Mondragon, A., Klug, A., & Regenmortel, M. H. V. (1984) *Nature (London) 311*, 123–126.
Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697–701.

Articles

Purification and Characterization of a Serine Protease (Esterase B) from Rat Submandibular Glands†

Madhu Khullar, Gloria Scicli, Oscar A. Carretero, and A. Guillermo Scicli*

Hypertension Research Division, Henry Ford Hospital, Detroit, Michigan 48202

Received April 9, 1985; Revised Manuscript Received October 4, 1985

ABSTRACT: A new protease has been purified to homogeneity from rat submandibular gland homogenate by using DEAE-Sephadex chromatography, chromatofocusing, aprotinin–Sepharose affinity chromatography, and high-performance liquid chromatography. The enzyme has been named esterase B, since it represents the second major esterolytic peak on DEAE-Sephadex chromatography of submandibular gland homogenate. It is an acidic protein (p*I* = 4.45) with an apparent molecular weight of 27 000. It is heat-stable and has an optimum pH of 9.5. Esterase B hydrolyzed the synthetic substrates tosyl-L-arginine methyl ester and Val-Leu-Arg-*p*-nitroanilide (S2266). It also cleaved dog plasma kininogen to produce a kinin, identified as bradykinin on reverse-phase high-performance liquid chromatography. Esterase B, however, is only a weak kininogenase, since it had only 5% of the kininogenase activity of equimolar concentrations of glandular kallikrein and had no effect on rat mean blood pressure or on the isolated rat uterus. Esterase B activated plasminogen and had caseinolytic activity. It was inhibited by aprotinin, soybean trypsin inhibitor, lima bean trypsin inhibitor, phenylmethanesulfonyl fluoride, antipain, leupeptin, and *p*-tosyl-L-lysine chloromethyl ketone. On double immunodiffusion, when reacted with kallikrein and tonin antisera, esterase B showed partial identity with kallikrein but not with tonin. On immunoelectrophoresis against kallikrein antisera, esterase B formed a precipitin arc at a position different from that of kallikrein. Esterase B appears to be a trypsin-like serine protease having some homology with glandular kallikrein.

Esterolytic activity in mammalian salivary glands was first demonstrated by Hüfner in 1873, as cited in Willslatter (1924). While this activity varies from species to species, rats have remarkably high esterolytic activity in their salivary glands (Junqueira & Fapa de Moraes, 1965). A number of proteases with esterolytic activity have been identified in rat salivary glands (Riekkinen & Hopsu-Havu, 1965; Minato et al., 1967). Riekkinen and co-workers partially purified and characterized two of these esterases, namely, salivain and glandulain (Riekkinen et al., 1966, 1967). However, Brandtzaeg and co-workers reported that salivain was a mixture of kallikrein and an unidentified protease (Brandtzaeg et al., 1976). Although rat salivary gland kallikrein (Brandtzaeg et al., 1976) and tonin (Boucher et al., 1974) have been well characterized, there is very little information available regarding other salivary gland proteases. Here, we report the purification and characterization of a protease from rat salivary gland homogenate. This enzyme is a weak kininogenase that belongs to the serine protease family and has properties distinctly different from those of glandular kallikrein, glandulain, salivain, and tonin.

Materials and Methods

The following reagents were obtained from commercial sources: DEAE-Sephadex A-50, Polybuffer anion exchanger (PBE 94), Polybuffer 74, low molecular weight protein markers (LMWK), and isoelectric pH markers (Pharmacia Fine Chemicals); tosyl-L-arginine [^{3}H]methyl ester ([^{3}H]-TAME)[1] (Amersham Corp.); acrylamide, *N*,*N*′-methylene-

† This work was supported by NIH Grants HL 21092 and HL 28982.
* Address correspondence to this author.

0006-2960/86/0425-1851$01.50/0